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IgE to epitopes of Ara h 2 enhance the diagnostic accuracy of Ara h 2-specific IgE

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Abstract

Background: Understanding the discrepancy between IgE sensitization and allergic reactions to peanut could facilitate diagnosis and lead to novel means of treating peanut allergy.

Objective: To identify differences in IgE and IgG4 binding to peanut peptides between peanut-allergic (PA) and peanut-sensitized but tolerant (PS) children.

Methods: PA (n = 56), PS (n = 42) and nonsensitized nonallergic (NA, n = 10) patients were studied. Synthetic overlapping 15-mer peptides of peanut allergens (Ara h 1-11) were spotted onto microarray slides, and patients' samples were tested for IgE and IgG4 binding using immunofluorescence. IgE and IgG4 levels to selected peptides were quantified using ImmunoCAP. Diagnostic model comparisons were performed using likelihood-ratio tests between each specified nominal logistic regression models.

Results: Seven peptides on Ara h 1, Ara h 2, and Ara h 3 were bound more by IgE of PA compared to PS patients on the microarray. IgE binding to one peptide on Ara h 5 and IgG4 binding to one Ara h 9 peptide were greater in PS than in PA patients. Using

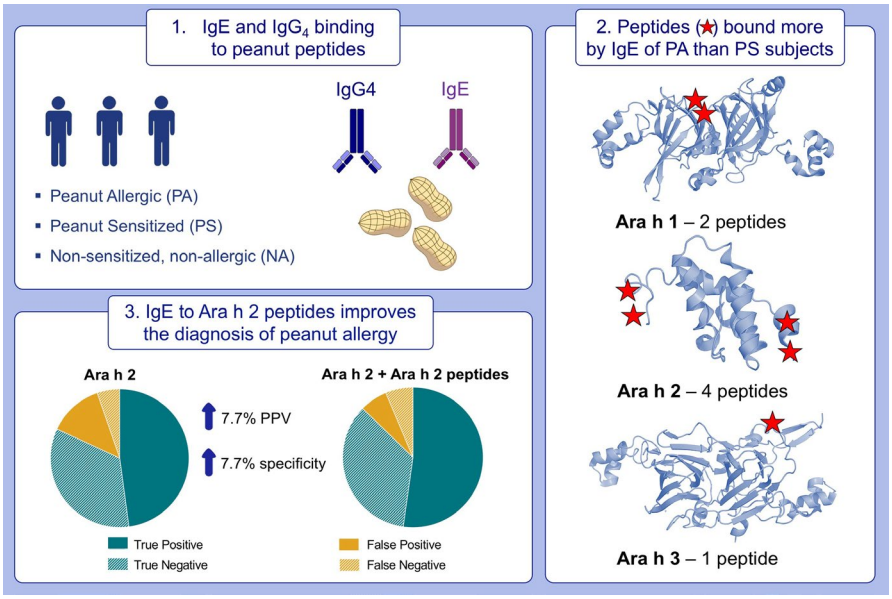
Abbreviations: DBPCFC, double-blind placebo-controlled food-challenge; FBR, foreground-to-background ratio; MAT, mast cell activation test; MBP, maltose-binding protein; OFC, oral food challenge; PA, peanut-allergic; PPV, positive predictive value; PS, peanut-sensitized tolerant; P-sIgE, peanut-specific IgE; RBL, rat basophilic leukemia; SPT, skin prick test. Barbosa-Morais and Hurlburt are contributed equally to this work.

ImmunoCAP, IgE to the Ara h 2 peptides enhanced the diagnostic accuracy of Ara h 2-specific IgE. Ratios of IgG4/IgE to 4 out of the 7 peptides were higher in PS than in PA subjects.

Conclusions: Ara h 2 peptide-specific IgE added diagnostic value to Ara h 2-specific IgE. Ability of peptide-specific IgG4 to surmount their IgE counterpart seems to be important in established peanut tolerance.

KEYWORDS

Ara h 2, diagnosis, epitopes, food allergy peanut allergy



GRAPHICAL ABSTRACT

This study identifies differences in IgE and IgG4 binding to peanut peptides between peanut-allergic and peanut-sensitized children. Peanut peptide microarray analyses reveal that seven peptides on Ara h 1, Ara h 2 and Ara h 3 are bound more by IgE of peanut-allergic compared to peanut-sensitized patients. Measuring IgE to the Ara h 2 peptides in combination with Ara h2 enhances the diagnostic accuracy of Ara h 2-specific IgE.

Abbreviation: PPV, positive predictive value

1 | INTRODUCTION

Allergen-specific IgE is necessary but not sufficient for the development of allergic reactions to a food allergen. Thus, IgE sensitization to foods can often be identified without proven clinical relevance. For instance, in the case of peanut, about 11.8% of school-age children in the United Kingdom have detectable specific IgE to peanut and only 2.6% are actually peanut-allergic as confirmed by double-blind placebo-controlled food-challenge (DBPCFC).[1] This discrepancy between allergic sensitization and clinical reactivity poses diagnostic difficulties and raises fundamental questions about the mechanisms of food allergy and oral tolerance. If IgE binds to the allergen in immunoassays to peanut, why is it not able to cause effector cell activation in the majority of patients?

We have been addressing two nonmutually exclusive hypotheses to explain the discrepancy between the presence of peanut-specific IgE (P-sIgE) and peanut allergy. The first hypothesis is that there may be differences in the characteristics of allergen-specific IgE between peanut-allergic (PA) and peanut-sensitized but tolerant (PS) patients. We have previously shown that, at the population level, PA patients tend to have higher levels of P-sIgE and to have IgE directed to the major peanut allergens, Ara h 1, Ara h 2, and Ara h 3.[2] However, there is a large overlap in the distribution of specific IgE levels in PA and PS patients. At the individual level, many cases can be found of PS patients who eat peanut without developing any symptoms and have relatively higher levels of P-sIgE compared with PA patients who develop allergic reactions, often severe, when exposed to peanut. This is also the case when considering specific IgE to Ara h 2, which has proved to be particularly discriminative between allergic and tolerant

individuals.[3] Refining IgE specificity at the epitope level may clarify this further; with existing experimental approaches using microarray and other platforms allowing to primarily identify linear epitopes.[4-7] Various groups have studied IgE epitopes on peanut allergens; however, most studies focused on searching for peanut epitopes bound by IgE of peanut-allergic patients and were limited to Ara h 2 or at most Ara h 1, Ara h 2, and Ara h 3. Ours was the first study to test IgE and IgG4 binding to all peanut allergens and to analyze the differential binding between PA and PS who were mostly sensitized to peanut major allergens, some able to cause allergic symptoms (as in the case of PA patients) and some not (as in the case of PS subjects).

The second hypothesis to explain the discrepancy between sensitization and allergy is that PS patients may have a peanut-specific antibody, such as IgG4, that are able to interfere with the allergen-IgE interaction. We previously showed that the levels of IgG4 to peanut were higher in PS compared with PA patients but it was the relative amount of IgG4 compared with IgE in individual patients, that is, the IgG4/IgE ratio, that enabled a clearer distinction between PA and PS patients with PS patients having higher IgG4/IgE ratios for peanut, Ara h 1, Ara h 2, and Ara h 3.[2] Depletion of IgG4 antibodies from plasma samples with detectable IgE to the major peanut allergens that would otherwise be predictive of peanut allergy partially restored mast cell activation, which supported a role of IgG4 in the absence of an effector cell response characteristic of peanut tolerance.[2]

A complete understanding of the mechanisms by which IgE and allergen may or may not be able to elicit effector cell activation that is responsible for the clinical manifestations of allergic disease requires a molecular approach. In this study, we aimed to identify the epitope specificities of IgE and IgG4 in PA and PS children to improve our understanding of the interplay between IgE and IgG4 in modulating peanut allergen-induced effector cell responses and consequent allergic reactions.

2 | METHODS

2.1 | Study procedures

Patients undergoing diagnostic evaluation for suspected peanut allergy were studied. The study was approved by the South East London 2 Research Ethics Committee. Written informed consent was obtained from the parents of all participants. Study participants underwent detailed clinical assessment, skin prick testing, specific IgE and IgG4 testing, and oral peanut challenges, as previously described.[8] Skin prick testing was performed using a commercially available peanut extract (ALK-Abelló). Serum-specific IgE and IgG4 to peanut were measured using an immunoenzymatic assay (ImmunoCAP, Thermo Fisher). Specific IgE to 112 allergens was determined using the ISAC microarray (Thermo Fisher).

Study participants were grouped as PA, PS and nonsensitized nonallergic (NA). Peanut allergy was confirmed by a positive oral food challenge (OFC) or by the combination of reported immediate-type allergic reactions to peanut and skin prick test (SPT) and/or P-sIgE greater or equal than the validated 95% positive predictive value (PPV) cutoffs of 8 mm and 15 kU/L, respectively. Peanut allergy was excluded by a negative OFC or the ability to eat ≥ 4 g of peanut protein twice a week, as assessed by a validated peanut consumption questionnaire. Peanut-sensitization was defined by a wheal size of peanut SPT ≥ 1 mm and/or specific IgE ≥ 0.10 KU_A/L. Out of the 108 patients studied, 78 (72%) had OFC, 68 had DBPCFC, and 10 had open OFC for logistical reasons, as previously reported. Twelve (15%) of OFC were positive, and 66 (85%) were negative.

2.2 | Peanut peptide microarray

Synthetic overlapping 15-mer peptides representing the entire amino acid sequence of ten peanut allergens Ara h 1 to 11 (Table S1), offset by 5 amino acids, were synthesized and printed in triplicate onto microarray slides (JPT Peptide Technologies GmbH, Berlin, Germany) together with peptides from other nuts and plant foods (see Table S2 for a full list of the peptides tested). Slides were placed in individual chambers of a HS400 Pro™ (Tecan, San Jose, CA) and blocked in filtered Superblock (Thermo, Rockford, IL) for 30 minutes at room temperature (RT). Following a wash with tris-buffered saline containing Tween-20, patients' plasma was injected and incubated at 4°C overnight. Slides were sequentially washed and incubated with mouse anti-human IgE and Cy3-conjugated goat anti-mouse IgG (both Life Technologies, Grand Island, NY). Slides were scanned using GenePix-4000B and the software GenePix-Pro7. The same slide was then re-blocked with Superblock and sequentially washed as above and incubated with rabbit-anti-human IgG4 (Abcam, Cambridge, MA) and anti-rabbit IgG Alexa Fluor (Life Technologies, Grand Island, NY) at RT for 30 minutes. The slides were washed and dried before scanning as above. IgE binding was measured by the Cy3, green fluorescence at 532 nm, and IgG4 binding by Alexa Fluor red fluorescence at 635 nm wavelength.

2.3 | Microarray data analysis

Scanning slides with GenePix Pro 7 (GP7) software-generated multilayer TIFF files which were analyzed by GP7 to generate GPR data files. These were read into the statistical software environment *R*, where all statistical analyses were done.[9] Quality assessment, preprocessing and differential binding analysis of the microarray data were performed using tools included in the *limma* package[10] available through the Bioconductor project.[11] Preprocessing of data comprised log-subtraction of mean background for each probe, mean-summarization of replicate probes followed by single-channel quantile normalization between arrays for contrasts involving only one antibody, and probe-level two-channel loess-normalization within arrays before computing IgG4/IgE ratios. IgE and IgG4 binding was expressed as the base 2 logarithm of the foreground to background ratio (FBR). See methods' section of the online repository for more details.

2.4 | Identification of the epitopes in the 3D structure of the allergens

The locations of the peptides in the 3D structures of the allergens, as deposited in the RCSB Protein Data Bank, were identified and visualized using PyMOL.[12]

2.5 | Quantification of specific IgE and IgG4 to peptides

Unblocked peptides (JPT Peptide Technologies GmbH, Berlin, Germany) were conjugated to the solid phase of ImmunoCAP by Thermo Fisher (Uppsala, Sweden). IgE and IgG4 binding was quantified using the Phadia 100 following the manufacturer's instructions. IgG4/IgE ratios were calculated as previously described by converting IgG4 levels from milligrams per liter to nanograms per milliliter and the peanut-specific IgE levels from kilo unit per liter to nanograms per milliliter with the use of the formula log10((IgG4 × 1000) ÷ (IgE × 2.4)).[2,13] Diagnostic model comparisons were performed using likelihood-ratio tests between each specified nominal logistic regression models using SAS version 9.4 and JMP Pro 14.

3 | RESULTS

3.1 | Study population

Plasma samples of patients consecutively and prospectively recruited were tested on a peanut peptide allergen microarray. Demographic and clinical data can be found in Table S3. Most patients were sensitized to the three major peanut allergens Ara h 1-3 (Table S4). Data of the 89 patients for whom there were results for both IgE and IgG4 binding following appropriate quality control (53 PA, 27 PS and 9 NA) were analyzed and compared between PA and PS patients. Total IgE (P = .402) and peanut-specific IgG4 (P = .122) were not significantly different between the 2 groups. PA patients had higher sIgE to peanut, (P < .001), Ara h 1 (P = .007), Ara h 2 (P < .001), and Ara h 3 (P = .017) than PS patients. Forty-six percent of patients assessed on ISAC were sensitized to Ara h 6 (84% of PA and 10% of PS) and 22% were sensitized to profilins (18% of PA and 33% of PS) with 13% being sensitized to Phl p 12 (6% of PA and 25% of PS)—data not shown.

3.2 | Ara h 1, Ara h 2, and Ara h 3 peptides were differentially bound by IgE of peanut-allergic and peanut-sensitized but tolerant patients

Various peanut peptides were able to bind IgE of peanut-sensitized patients, including both PA and PS patients (Figure S1). When analyzing the differences between PA and PS patients, 7 peptides associated with the major peanut allergens Ara h 1, Ara h 2, and Ara h 3 emerged in the differential binding analyses as having a higher degree of IgE binding in PA compared with PS patients (Figure 1, Table 1, and Figure S2). Peptide 10, on Ara h 5 (AA51-65) was bound preferentially by IgE of PS than by IgE of PA patients (Table 1). There was a positive association between IgE to the Ara h 5 peptide and IgE to peptides from Ara h 8 (Table S5). IgE binding to peptides from other peanut allergens was not significantly different between PA and PS patients.

3.3 | All peptides identified on Ara h 1, Ara h 2, and Ara h 3 were located on the surface of the allergens and thus were susceptible to antibody binding

The identified Ara h 1, Ara h 2, and Ara h 3 peptides were located on the surface of the allergens in structurally disordered or partially disordered loop regions (Figure 2). The Ara h 1 peptides (peptides 7 and 8) overlapped by 10 amino acids, ranging between amino acid 85 and 105 of the allergen, and were located in a part of the protein that is absent from the crystal structure and was predicted as disordered. Two of the Ara h 2 peptides, peptide 1 (AA61-75) and peptide 3 (AA81-95) were located on a flexible loop in a partially disordered region. The other two overlapping Ara h 2 peptides, peptide 2 (AA26-40) and peptide 4 (AA31-45), consisted of parts of two helices linked by a loop, located in the N-terminal region close to the link to maltose-binding protein (MBP) with which Ara h 2 was expressed and crystallized as a fusion protein.[14] The peptide identified in Ara h 3, peptide 9 (AA324-338) was located on a loop of a partially disordered region in an exposed part of the protein crystal structure.

3.4 | Quantification and diagnostic utility of IgE to the 7 peptides using ImmunoCAP

In order to validate our findings, we quantified IgE levels using the ImmunoCAP technology to the 7 peanut peptides that were bound more by IgE of PA than by IgE of PS on the microarray (Figure 3 and Figure S3). The differences in IgE binding to the peanut peptides between PA and PS patients were independent of their peanut-specific IgE levels (Figure S4). Specific IgE to the individual allergen components was detectable both in PA and in PS subjects (Table S3) and was generally related to specific IgE to the peptides from the respective allergen in both PA and PS subjects (Figures S5 and S6).

Considering the utility of ImmunoCAP to peptides to discriminate between peanut-allergic and nonallergic subjects among sensitized individuals, specific IgE to the four Ara h 2 peptides showed good diagnostic discrimination and enhanced the accuracy of Ara h 2-specific IgE (Figure 4). Specific IgE to peptides from Ara h 1 or Ara h 3 did not offer advantage over the respective allergen-specific IgE (Figures S7 and S8). Specific IgE to peanut was inferior to specific IgE to Ara h 2 (Figure S9).

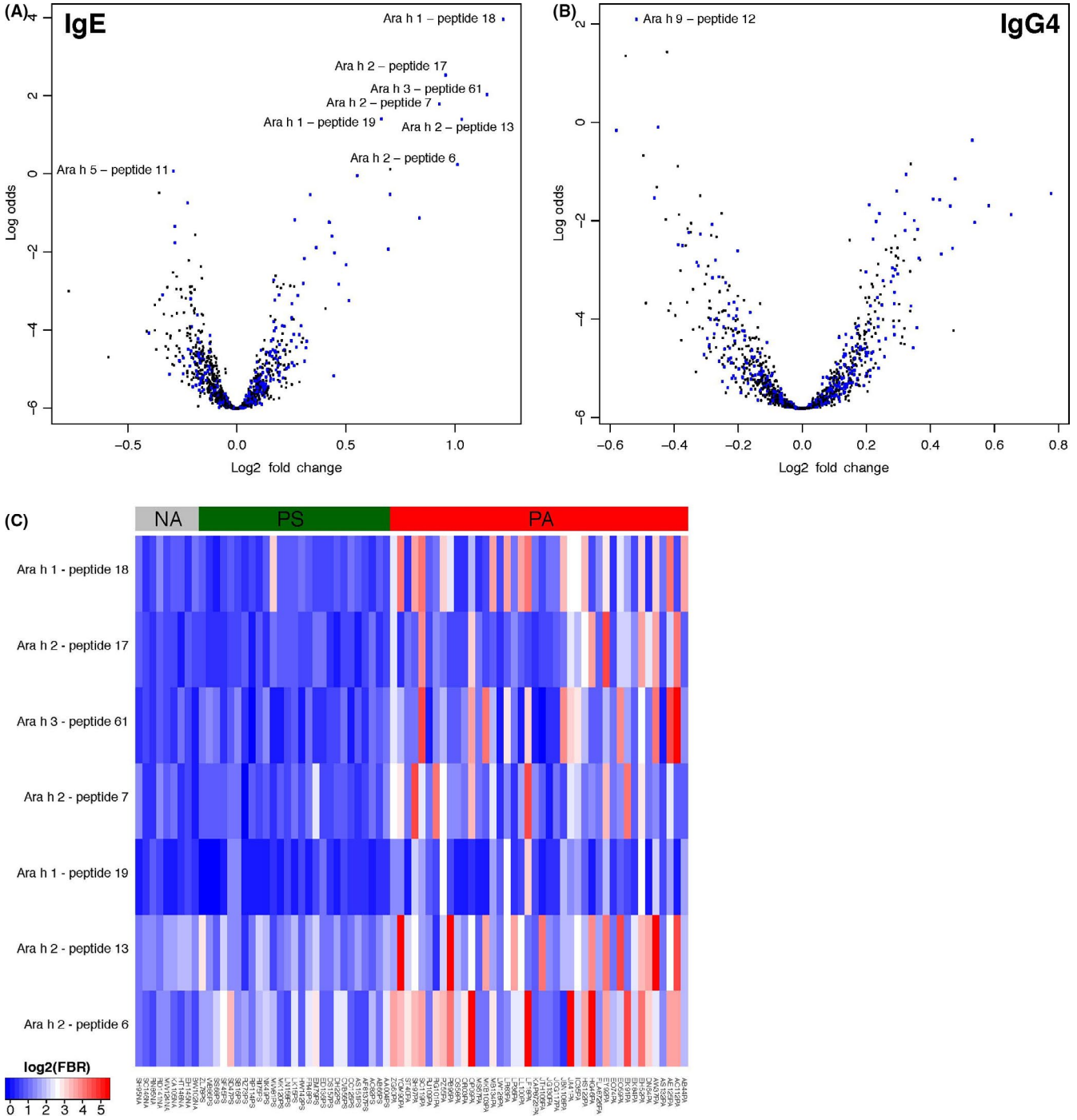


FIGURE 1 Volcano plots for (A) IgE and (B) IgG4 binding to peanut peptides between peanut-allergic (PA) and peanut-sensitized tolerant (PS) patients. The x-axis represents the log2 fold changes of average foreground-to-background ratio between PA and PS patients. The y-axis represents the empirical Bayes log-odds (B) of differential binding between PS and PA. Peanut peptides (blue dots) that are differentially bound between the groups (B > 0, ie, odds of differential binding higher than those of no effect, with FDR < 0.05) are named in the figure. (C) Heat map of IgE binding to peptides significantly more bound in PA (in red) than PS (in green) patients (NA patients are shown in grey for comparison) expressed as the binary logarithm of foreground-to-background ratio (log2(FBR))

3.5 | Similarity of IgE and IgG4 binding to peanut peptides

On the microarray, IgG4 binding to one Ara h 9 peptide, to one peptide from another lipid-transfer protein from peach Pru p 3 and from Gly m 5 from soya was greater in PS than in PA patients (Figure 1B, Table 1). For the remaining allergen peptides, there were no statistically significant differences in IgG4 binding between the two groups of patients. Overall, there was a strong association between peanut peptides bound by IgG4 and IgE both in PA and PS patients (Figure S10A). The number of peanut peptides bound by IgG4 and IgE was similar between the two groups of patients (Figure S10B); however,

TABLE 1 Peanut peptides differentially bound by IgE or IgG4 of peanut-allergic and peanut sensitized but tolerant patients

Antibody isotype	Allergen	Peptide name	Peptide name based on position in the protein sequence	Peptide sequence	logFC	B	% recognition PS (max NA)	% recognition PA (max NA)
IgE	Ara h 1	Peptide 7	Peptide 18	SPPGERTRGRQPGDY	1.22	3.96	3.70	54.76
	Ara h 2	Peptide 1	Peptide 17	RDPYSPSPYDRRGAG	0.96	2.53	18.52	54.76
	Ara h 3	Peptide 9	Peptide 61	EDEYEYDEEDRRRGR	1.15	2.03	33.33	64.29
	Ara h 2	Peptide 2	Peptide 7	RRCQSQLERANLRPC	0.93	1.79	3.70	40.48
	Ara h 1	Peptide 8	Peptide 19	RTRGRQPGDYDDDRR	0.66	1.41	11.11	50.00
	Ara h 2	Peptide 3	Peptide 13	GRDPYSPSQDPYSPS	1.03	1.40	25.93	61.90
	Ara h 2	Peptide 4	Peptide 6	ELQGDRRCQSQLERA	1.01	0.23	59.26	76.19
	Ara h 5	Peptide 10	Peptide 11	MNDFAEPGSLAPTGL	−0.29	0.07	44.44	11.90
IgG4	Ara h 9	Peptide 11	Peptide 12	GSLHGLNQGNAAALP	−0.52	2.09	18.52	0.00
	Gly m 5	Peptide 12	Peptide 6	QHGEKEEDEGEQPRP	−0.42119	1.43	29.63	4.76
	Pru p 3	Peptide 13	Peptide 12	GAVKGINPGYAAALP	−0.55018	1.35	11.11	2.38

Note: Probes are ranked inversely by the log-odds of being differentially bound between PS and PA (positive log2 fold changes (logFC) express greater binding by IgE of PA patients; negative logFC expresses greater binding by IgE or IgG4 of PS patients). B, empirical Bayes statistic which gives the logarithm (natural base) of the ratio between the odds of a peptide being differentially bound between PA and PS and the odds of not being differentially bound. B > 0 (ie, odds of differential binding higher than those of no effect, with false discovery rate (FDR) < 0.05) was considered statistically significant.

overall the ratio of IgG4/IgE to peanut peptides was higher in PS than in PA patients (Figure S11).

Using ImmunoCAP, we were able to quantify the levels of antibodies directed to specific peptides and to calculate with precision the relative amounts of IgE and IgG4. IgG4 levels to any of the 7 peptides were not significantly different between PA and PS. IgG4/IgE ratios were higher in PS than in PA patients for antibodies directed to peptides 1, 3, and 4 of Ara h 2, as well as to peptide 9 of Ara h 3 (Figure 5). Interestingly, we observed correlation of peptide-specific IgE and IgG4 with age (Figure S12).

4 | DISCUSSION

The discrepancy between the presence of P-sIgE and IgE-mediated allergic reactions to peanut is intriguing in that patients with similar levels of P-sIgE and even Ara h 2-specific IgE can have different clinical outcomes, some being PA and some being able to eat peanut without developing any symptoms. To explore the underlying reasons for this discrepancy, we have tested PA and PS patients for IgE and IgG4 binding to 15-mer peptides covering the sequence of all peanut allergens known at the time when the microarray was generated. We identified four Ara h 2 peptides, two Ara h 1 peptides and one Ara h 3 peptide that were bound preferentially by IgE of PA than by IgE of PS. One peptide of the profilin Ara h 5 was bound preferentially by IgE of PS. Quantification of IgE and IgG4 to selected peptides using ImmunoCAP technology revealed that specific IgE to the Ara h 2 peptides showed very good diagnostic utility and taken together with IgE to Ara h 2 were the best serologic marker for peanut allergy and better than Ara h 2-specific IgE alone. Additionally, ImmunoCAP allowed precise calculations of IgG4/IgE ratios to individual peptides, which were higher in PS than in PA patients, suggesting that the balance of IgG4 and IgE is important in established peanut tolerance.

For the first time, we have quantified IgE to peanut peptides that were discriminative between allergy and tolerance using ImmunoCAP technology and determined the diagnostic value of this approach. Importantly, we were able to show that the differences in IgE binding to the peptides between PA and PS were independent of the level of peanut-specific IgE. The peptides we have identified as most discriminative between PA and PS status overlap with some of the IgE-binding epitopes previously reported in pioneering studies in which IgE binding was assessed using samples from PA patients.[14-20] However, few studies have looked at the comparison of epitope specificity between PA and PS patients like ours.[21,22] The peptides we identified align totally or partially with epitopes reported by Lin et al[22]. Four peptides were particularly important to differentiate PA from PS using a machine learning method, decision tree, and support vector machine in the latter study[22]: two peptides on Ara h 2 which overlap with two of the peptides we identified and one peptide on Ara h 1 and one peptide on Ara h 3, which in turn are different from those that we have identified. There are some differences in the methodology used that could explain the different findings; for

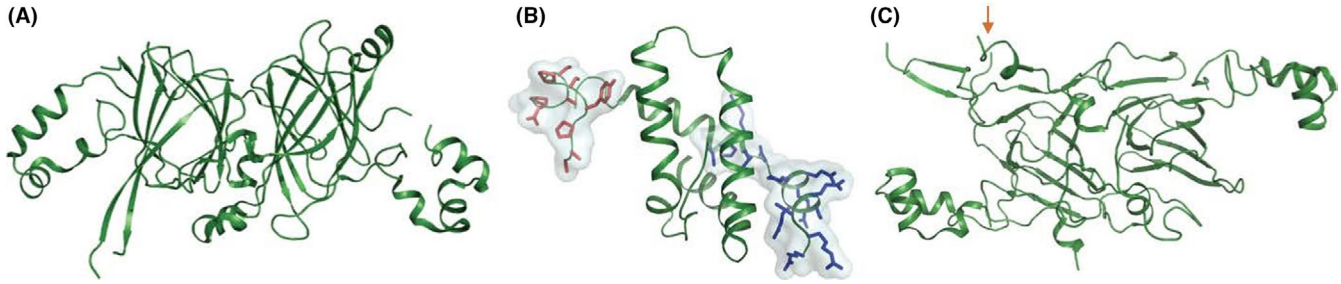


FIGURE 2 Peptides indicated on the X-ray structures for (A) Ara h 1 (B) Ara h 2 and (C) Ara h 3. The two overlapping peptides (7 & 8) on Ara h 1 are not shown as they are present in a disordered N-terminal region that has been truncated in the crystal structure. The four peptides for Ara h 2 are shown as sticks in red (peptides 1 & 3) and blue (overlapping peptides 2 & 4). Only the ordered residues are shown; most of these peptide residues are in flexible/disordered loop regions. The peptide for Ara h 3 (peptide 9) is present in the disordered loop region of the structure that is indicated by an arrow. The PDB codes for the structures used to generate the figures were as follows: 3S7I (for Ara h 1), 3OB4 (for Ara h 2), and 3C3V (for Ara h 3)

instance, Lin et al[22] used 15-mer peptides with an offset of 3 amino acids and only 9% of PS and 13% of PA had IgE to Ara h 1 or Ara h 3 and 4% of PS and 74% of PA had IgE to Ara h 2; whereas in our study, we used 15-mer peptides with an offset of 5 amino acids and the majority of patients both from PA and PS groups were sensitized to all three major peanut allergens (see Table S4).

We tested for all peanut allergens known at the time of generation of the microarray. Going beyond testing for the well-studied major allergens Ara h 1, Ara h 2, and Ara h 3 enabled us to explore the importance of allergens that are not commonly tested for. The fact that the peptides that are bound preferentially by IgE of PA than by IgE of PS are located on Ara h 1, Ara h 2, and Ara h 3 is a confirmation that these major allergens are indeed the most important in peanut allergy. More recently, oleosins[23] and defensins[24] have been described in peanut and may also be important; however, IgE to these lipophilic allergens is probably not as dominant as IgE to Ara h 1, Ara h 2, and Ara h 3, given that it is uncommon to find PA patients with negative SPT or specific IgE, which are tests that use extracts that are defatted and thus lack lipophilic proteins.

The crystal structures of Ara h 1,[25,26] Ara h 2,[14] and Ara h 3[27] have been totally or partially solved; thus, we were able to establish the location of the relevant peptides in these 3D structures. The identified peptides were all located on the surface of peanut allergens, in structurally disordered or partially disordered loop regions, accessible to antibody binding. Two Ara h 2 peptides adopt a partly alpha-helical conformation in the fusion protein; however, in Ara h 2 alone, they might be more flexible. A crystal structure of Ara h 2 on its own, without MBP, would be necessary to clarify this. Epitopes located on the surface of the allergens are indeed particularly susceptible to antibody binding, including receptor-bound IgE on the membrane of mast cells and basophils, and thus are more likely to be able to elicit effector cell activation and allergic symptoms. Finding mainly epitopes located on the surface of the allergens could indicate that the linear epitopes were part of epitopes formed by parts of the protein that are close in the 3D structure but distant in the protein sequence (ie, conformational epitopes). Because we used linear short peptides to test for IgE and IgG4 binding, we will not have been able to detect conformational epitopes. Conformational epitopes are likely to be important in IgE binding, particularly to allergens that are labile to heating and digestion, such as pollen-cross-reactive allergens like Ara h 8 and Ara h 5. In our study, a peptide from the peanut profilin Ara h 5 was identified as being bound preferentially by IgE of PS than by IgE of PA. Profilins are pan-allergens with unclear clinical relevance in peanut allergy.[28] Profilin is likely to be an important cause of false-positives in P-sIgE testing, and its importance is probably underestimated as specific IgE to peanut profilin is not commercially available and thus is not usually tested in isolation. Additional characteristics of IgE, apart from epitope specificity, may contribute to the discrepancy between sensitization and clinical allergy, notably differences in diversity and affinity of IgE antibodies for the peanut epitopes and the spatial distribution of these epitopes.[29]

We moved from a semi-quantitative microarray to the quantitative method ImmunoCAP to show the statistical, biological, and clinical impact of the epitopes contained in the peptides we identified. The ImmunoCAP technology allowed us to quantify the levels of IgE to the peptides and showed that IgE to the Ara h 2 peptides improved the diagnostic utility of IgE to Ara h 2, which could have direct practical clinical implications. It was impressive that IgE to Ara h 2 peptides alone had good diagnostic performance and could enhance the accuracy of Ara h 2-specific IgE, a diagnostic test that is already able to discriminate very well peanut-allergic from non-allergic individuals.[3,8] Even if the number of subjects with equivocal levels of Ara h 2-specific IgE is a small proportion of the population tested, it is clinically relevant for those individuals and could enable us to reduce the number of patients we need to subject to an oral peanut challenge. The combination of IgE to the four Ara h 2 peptides can improve the diagnostic utility of Ara h 2-specific IgE in equivocal cases and, to make this approach more practical, could be provided as a single test in the ImmunoCAP platform in the future, following additional validation to confirm that such approach would not lead to a loss in sensitivity.

The alternative hypothesis we explored related to IgG4 interfering with the interaction between IgE and the allergen. No differences in IgG4 binding to peanut peptides could be identified on the microarray between PA and PS patients, except for one Ara h

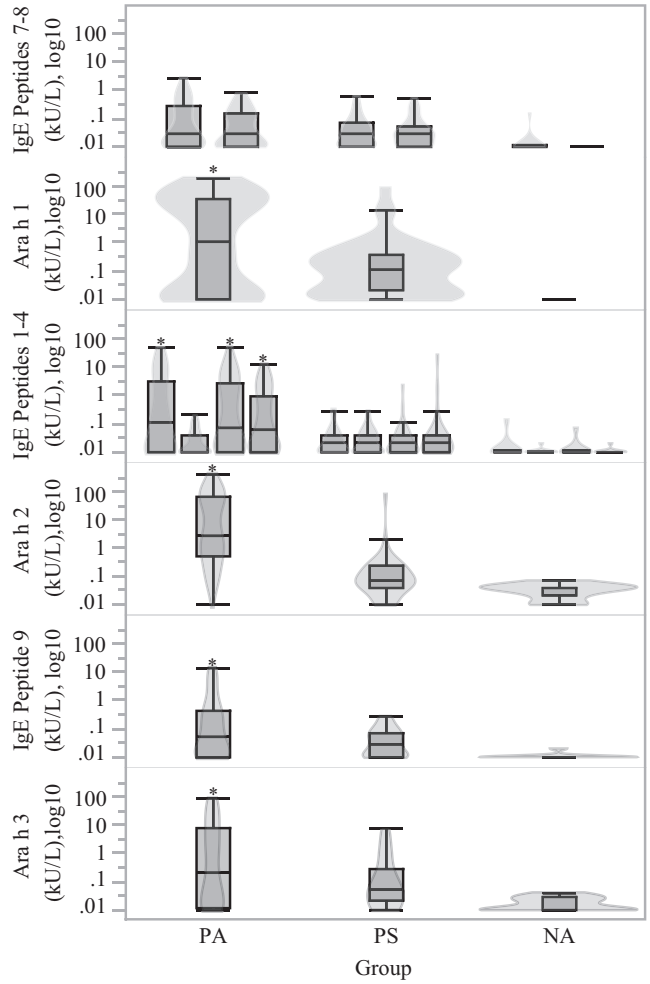


FIGURE 3 Box and violin plots of specific IgE to individual peanut peptides and the respective peanut allergen. Peptides are grouped and displayed sequentially from left to right overtop each of their respective peanut allergen by peanut-allergic (PA), peanut-sensitized tolerant (PS) and nonsensitized nonallergic (NA) measured by ImmunoCAP (Thermo Fisher). Statistically significant comparisons ($P < .01$) between PA and PS are marked with an * above each box plot. The exact *P*-values are as follows for each IgE to the respective peptides and to Ara h1-h3: 1 (<.001), 2 (.543, 3 (<.001), 4 (.001), 7 (.100, 8 (.280), and 9 (.004), Ara h 1 (<.001), Ara h 2 (<.001), Ara h 3 (.002)

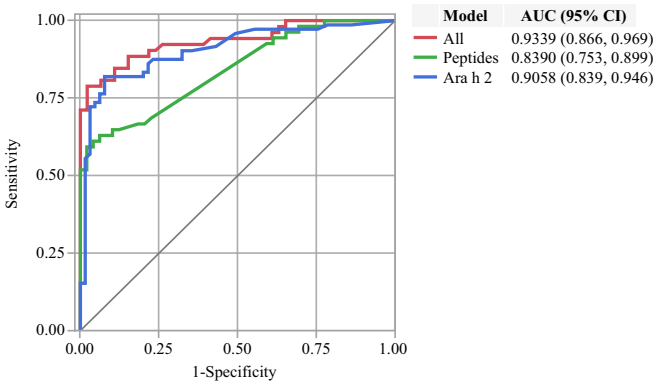


FIGURE 4 ROC curves for identifying peanut allergy using the following: 1. the combination of IgE to Ara h 2 and IgE to each of its peptides (1,2,3,4), labeled "All"; 2. Ara h 2-specific IgE alone (labelled "Ara h 2"), and 3. combination of specific IgE to the 4 Ara h 2 peptides (labeled "Peptides"). The hypothesis test that the AUC from all models are equal was rejected ($P < .0001$), and we made the following pair wise comparisons of the difference between the AUCs of each model with (95% CI), *P*-values using likelihood-ratio tests. All vs Peptides:.095 (.032,.158), $P = .003$. All vs Ara h2: .028 (.013, .043), $P = .0002$. Peptides vs Ara h2: −.067 (−.138,.004), $P = .0656$. All data were used for the model comparisons, and no imputation of missing data was performed. However, because each predictor had different amounts of missing data, the model comparisons used slightly different cohorts of participants

9 peptide. As IgE to Ara h 9 was very low in the studied patients and not significantly different between PA and PS patients, the clinical relevance of this finding is unclear. This peptide is predicted to adopt an exposed helix and loop structure, based on a model of Ara h 9 generated from the highly homologous structure of the pea lipid transfer protein (PDB ID: 2N81). Both PA and PS patients tended to produce IgE and IgG4 to the same peptides, as reported in a previous study of Ara h 2[7]; however, the relative amounts of specific IgE and IgG4 present were different with IgG4/IgE ratios prevailing in PS patients, as we previously showed at the level of the whole allergen.[2] As the microarray is semi-quantitative, and thus not an accurate method to precisely quantify the amount of IgE and IgG4 antibodies that bound each peptide, we quantified the levels of IgG4 to the peanut peptides that were distinct between PA and PS in the microarray using the ImmunoCAP technology. IgG4/IgE ratios were calculated as in previous studies[2] and were higher in PS than in PA patients for peptides 1, 3, and 4 of Ara h 2 and peptide 9 of Ara h 3. These findings support the role of the IgG4/IgE balance in established peanut tolerance.

Competition for binding to the peptides could potentially have interfered with the results of the microarray given that IgE and IgG4 binding were measured using the same slide for each patient, particularly in the PS group, similar to what occurs in other assays using microarrays.[30] However, the peptides were printed onto the slide in large amounts and in excess of what was expected to be bound by IgE; therefore, there should have been enough peptide to prevent saturation of the system and to allow enough antibody binding. As there was serial exposure to the antibodies used for detection of the IgE and the IgG4, controls were included in which the detection antibodies were reversed with anti-IgG4 being added first followed by anti-IgE; no significant differences were observed. Sera from non–peanut-allergic individuals with undetectable peanut-specific IgE and elevated total IgE were additionally used as negative controls. Human sera from nonallergic controls and chicken sera were also used to check for nonspecific binding.

In the future, we would like to integrate information about the exact location of epitopes, the distance between them, and their repetition and combination in the allergen structure, with the affinity of binding. Understanding the interplay between all these

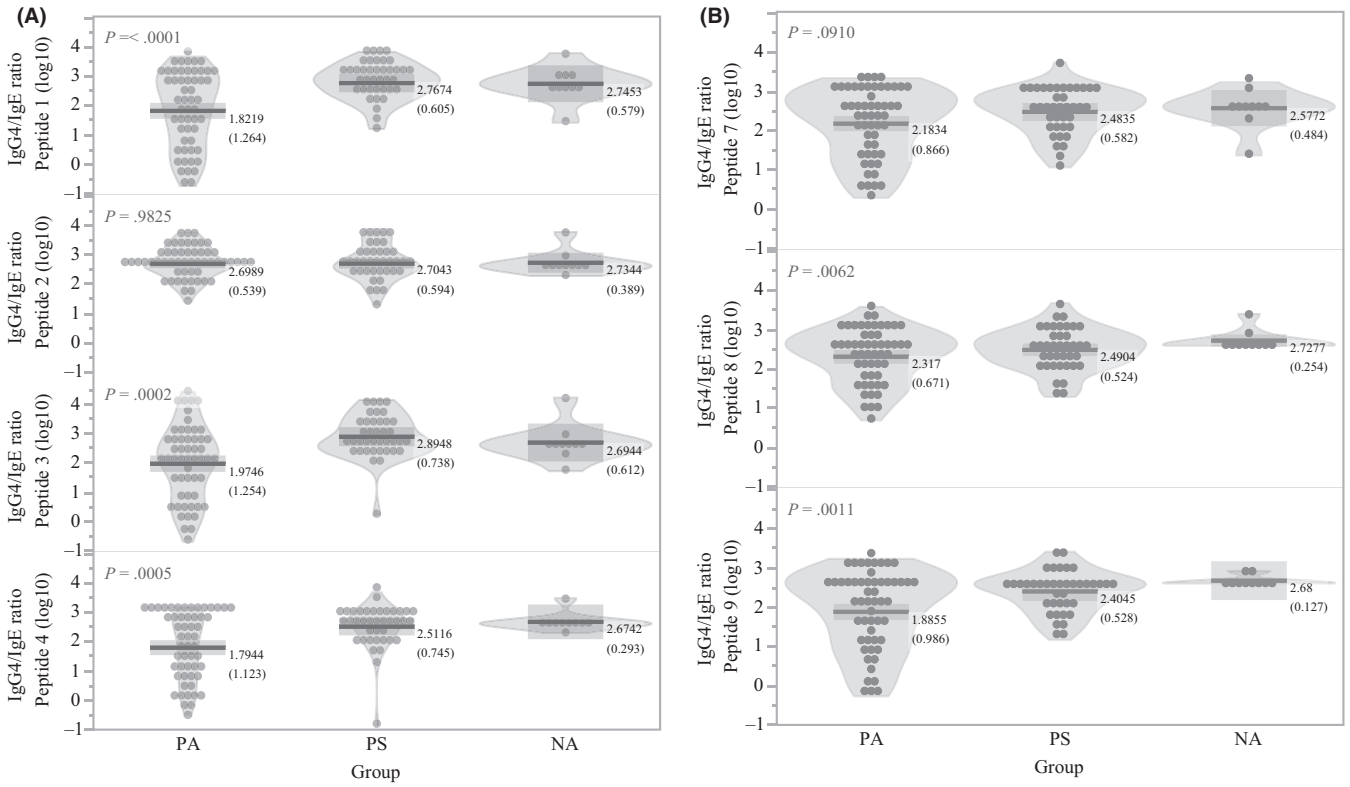


FIGURE 5 IgG4/IgE ratios to individual peptides from (A) Ara h 2 and (B) Ara h 1 and Ara h 3 in peanut-allergic (PA), peanut-sensitized tolerant (PS) and nonsensitized nonallergic (NA). IgE and IgG4 levels to the peptides were measured by ImmunoCAP and IgG4/IgE ratios were calculated following conversion of IgG4 levels from milligrams per liter to nanograms per milliliter and of IgE levels from kilo unit per liter to nanograms per milliliter using the formula log10((IgG4 × 1000)/(IgE × 2.4)). *P*-values are represented for the comparison across the three groups. For the comparison between PA and PS, the *P*-values are given in brackets for the respective peptides: 1 (<.001), 2 (.9627), 3 (<.001), 4 (<.001), 7 (.0564), 8 (.1651), and 9 (<.001)

factors could clarify what determines the ability of IgE and allergen to cause effector cell activation. Clarifying the mechanism by which PS patients do not react to peanut despite the presence of IgE could help to identify targets for novel curative treatments for peanut and other food allergies.

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CONFLICT OF INTEREST

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AUTHORS CONTRIBUTIONS

AFS designed the study, recruited the patients, led the experimental work, data analyses and interpretation of results, and wrote the manuscript; BKH, HC, and SJM designed and performed the microarray experiments; NLBM analyzed the microarray data; OH and MK performed the ImmunoCAP experiments; HTB performed statistical analyses of ImmunoCAP data; SR, LKJ, HJG, and BJS performed the structural analyses and advised on the experimental approach; GL contributed to study design, patient recruitment and interpretation of results; all authors contributed to the manuscript and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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